Both the 5S rRNA gene and the AT-rich flanks of *Xenopus laevis* oocyte-type 5S rDNA repeat are required for histone H1-dependent repression of transcription of pol III-type genes in *in vitro* reconstituted chromatin

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Received September 7, 1998; Revised and Accepted October 27, 1998

ABSTRACT

Incorporation of somatic histone H1 into chromatin during embryogenesis of *Xenopus laevis* results in repression of transcription of the oocyte- but not the somatic-type 5S rRNA genes. We showed earlier that a similar effect of the H1 observed in chromatin reconstituted on circular plasmids *in vitro* depends on its binding to the AT-rich flanks of the oocyte-type 5S rRNA gene. H1 binding results in stabilization of nucleosomes within the oocyte 5S rDNA repeat comprising the 5S rRNA gene with flanks and in reorganization of chromatin on the entire plasmid DNA. Performing *in vitro* transcription on reconstituted minichromosome templates carrying the oocyte 5S rRNA gene placed in different arrangements and at different distances from the AT-rich flanks, we now establish that the above effects of H1 observed upon its binding to the AT-rich sequences are absolutely dependent on the presence of the 120 bp oocyte 5S rRNA gene in its native position within the flanks. We also find that with the intact oocyte 5S rDNA repeat, the binding of H1 results in repression of transcription of both pol III- and pol II-type genes and that the transcriptionally inactive chromatin state spreads over a distance of at least a few nucleosomes.

INTRODUCTION

In eukaryotes the DNA molecules are packed into nucleosomes that are condensed into higher order chromatin structures. The presence of nucleosomes and their higher order structures can influence the transcription of genes. There exist a number of mechanisms that either release the chromatin-based constraint on transcription or induce transcriptionally repressed chromatin states (1). These mechanisms can be crucial in determining and maintaining specifically altered chromatin states as cells differentiate during development (2,3).

Histone H1 has a role in establishing the transcriptionally repressed chromatin state of defined sets of genes (reviewed in 2). The best documented case of this role is the involvement of H1 in developmental regulation of 5S rRNA gene expression in *Xenopus laevis*. In this organism there are two gene families of 5S rRNA genes: the oocyte 5S rRNA genes, consisting of 20 000 copies of the ‘oocyte’ repeat per haploid genome, and somatic 5S rRNA genes, consisting of 400 copies of the ‘somatic’ repeat per haploid genome. The somatic genes are active in growing oocytes, at the mid-blastula transition (MBT) and in somatic cells. The oocyte genes are active in growing oocytes up to the MBT and repressed in somatic cells. The major somatic form of *Xenopus* histone H1, which only occurs beginning from the MBT stage of embryonic development, acts as a specific repressor for the oocyte 5S rRNA genes (4,5). The oocyte-type 5S rDNA repeat in *X.laevis* comprises a 120 bp 5S rRNA gene placed within a few hundred base pair long, 76% A+T-rich flanks. The somatic-type repeat contains an almost identical 120 bp 5S rRNA gene surrounded by GC-rich flanks (6). It has been found that the repressed oocyte-type 5S genes, but not the active somatic-type genes, are incorporated into positioned nucleosomes *in vivo* (7).

In an earlier study (8), using a fully defined *in vitro* system of chromatin assembly on plasmid templates, we found that the AT-rich sequences surrounding the oocyte 5S rRNA gene enable H1 to realign nucleosomal core particles densely packed on the circular DNA. As this H1-mediated chromatin reorganization restricted the accessibility of the oocyte-type 5S rRNA gene to nucleases and prevented its transcription *in vitro*, we suggested that it could be the reason underlying the selective repression by H1 observed *in vivo*. If this was so, then the key factor determining the ability of H1 to repress transcription would be the presence of the AT-rich flanks surrounding the oocyte-type 5S rRNA genes and functioning as a specific H1-dependent ‘silencer’ sequence. To verify this hypothesis, we decided to determine in the present work the conditions that the AT-rich...
saturated bromide gradient followed by repetitive extraction with water-alkaline lysis method and purified twice on a CsCl/ethidium (template VII) (Fig. 4). All plasmids were prepared by the gene was cloned into the template was replaced with the RNA gene surrounded by the AT-rich flanks in the original DNA

Drosophila long designated IV contained the 5S rDNA cloned into the +426 of the v.4.0. In order to prepare templates VI and VII the region –861 to ScaI (template II) or ScaI (template III). The template PvuII site of the original template

flanks of the oocyte 5S rRNA gene and the rest of plasmid DNA, PCR amplification of the DNA fragments containing the AT-rich flanking sequences from pXlo excision of the oocyte-type 5S RNA gene surrounded by its native AT-rich flanking sequences (Fig. 2). In order to prepare templates II and III, template I was used for PCR amplification of the DNA fragments containing the AT-rich flanks of the oocyte 5S rRNA gene and the rest of plasmid DNA, but not the 5S rRNA gene itself. The PCR product, circularized by ligation of ends, contained the entire length of the AT-rich flanks in the form of a continuous DNA fragment. Into this DNA sequences from the AT-rich flanks. The results show that the complete X.laevis oocyte-type 5S rDNA repeat (a gene surrounded by its AT-rich flanks) is indispensable for effective transcriptional repression by H1. We also found that with the intact oocyte 5S rDNA repeat the effect of H1 is exerted on both pol III- and pol II-type genes and that the transcriptionally inactive chromatin structure spreads over a distance of at least a few nucleosomes.

MATERIALS AND METHODS
Preparation of histones and DNA

Core histones were isolated from chicken red blood cell nuclei as described (9). Histone H1 was derived from calf thymus by the method described (10). The concentration of histones was determined using the Bradford procedure with the BioRad Protein Assay (BioRad Laboratories Inc.). The concentration of histone H1 was calculated from absorption at 280 nm using an extinction coefficient of 2.0 for a 10 mg/ml solution of histone H1. Solutions of histones were stored in small aliquots frozen at –80°C. DNA templates used for minichromosome reconstitution were plasmid pBR327 derivatives which contained the AT-rich DNA sequences from the X.laevis oocyte-type 5S rDNA repeat, placed in different orientations with regard to the transcribed gene.

The original template (template I) was prepared by excision of the oocyte-type 5S RNA gene surrounded by its native AT-rich flanking sequences from pXloΔ3+176 plasmid (11) and subsequently cloning it into the HindIII site of plasmid pBR327. In order to prepare templates II and III, template I was used for PCR amplification of the DNA fragments containing the AT-rich flanks of the oocyte 5S rRNA gene and the rest of plasmid DNA, but not the 5S rRNA gene itself. The PCR product, circularized by ligation of ends, contained the entire length of the AT-rich flanks in the form of a continuous DNA fragment. Into this circular DNA, the PCR-amplified 5S rDNA was cloned into the ScaI site (template II) or PvuII site (template III). The template designated IV contained the 5S rDNA cloned into the ScaI site of the original template. In template V the 5S rRNA gene from the oocyte-type 5S rDNA repeat was replaced with the neutral 117 bp long ScaI–XmnI DNA fragment (Fig. 2). The PCR amplification conditions were set using the primer analysis software Oligo v.4.0. In order to prepare templates VI and VII the region –861 to +426 of the Drosophila Kruppel segmentation gene (Kr) in a pUC119 vector (12) was excised with Sall and XbaI from Drosophila Kruppel promoter DNA (Promega Corp.). The 5S RNA gene surrounded by the AT-rich flanks in the original DNA template was replaced with the Kr gene (template VI) or the Kr gene was cloned into the ScaI site of the original template (template VII) (Fig. 4). All plasmids were prepared by the alkaline lysis method and purified twice on a CsCl/ethidium bromide gradient followed by repetitive extraction with water-saturated n-butanol, dialysis against two changes of TE buffer and precipitation with ethanol (15). The concentration of DNA was measured spectrophotometrically at 260 nm using an extinction coefficient of 1.0 for a 50 µg/ml solution.

Reconstitution and analysis of minichromosomes

Minichromosomes were reconstituted by salt gradient dialysis (14). Defined amounts of supercoiled plasmid DNA and core histones were mixed in a final volume of 50 µl and treated as described (8). The addition of H1 was performed as described (8). Where indicated, the minichromosomes after nucleosome assembly were analysed by supercoiling assay using two-dimensional electrophoresis in 1.2% agarose (8).

In vitro transcription assay

Transcription extracts from mouse Ehrlich ascites tumour cells were prepared by the method of Dignam et al. (15). The reaction mixture for a standard in vitro transcription assay contained 12.5 mM HEPES (pH 7.7), 10% glycerol, 70 mM KCl, 0.5 mM DTT, 5 mM MgCl₂, 600 µM ATP, 600 µM CTP, 600 µM GTP, 60 µM UTP, 20 µM [α³²P]UTP (3000 Ci/mmol) (Amersham), 1 µg DNA template and 25 µl of a crude cell-free extract in a total volume of 50 µl. DNA templates were used in the form of naked DNA or reconstituted minichromosomes containing or lacking H1. Before the addition of the ribonucleoside triphosphates, the mixtures were pre-incubated with the transcription extract for 50 min at 30°C. The transcription reaction was carried on for 60 min at 30°C and was terminated by addition of an equal volume of solution containing 0.5% SDS, 0.1 M sodium acetate (pH 5.5) and 50 µg carrier yeast RNA. The 5S rRNA was isolated from the reaction mixture according to Dignam et al. (16), redissolved in deionized formamide, heated at 95°C for 3 min and loaded on a 12.5% polyacrylamide (acrylamide:bis-acrylamide 16:1) gel containing 4 M urea. Electrophoresis was carried out for 18 h at 4–5 mA and the radioactive gels were exposed to Fuji RX film. The product of the Kr gene prior to separation on a 1% agarose gel was treated with RNase-free DNase, transferred onto a nylon membrane (Hybond-N; Amersham) and hybridized at 68°C with a digoxigenin-labelled fragment of the Kr gene under the conditions described in the manufacturer’s protocol (Boehringer Mannheim).

RESULTS

In vitro transcription of reconstituted minichromosome templates containing the 5S rRNA gene displaced from its original AT-rich flanks

Previously we found that in a cell-free in vitro transcription system with reconstituted minichromosomes containing the oocyte- and somatic-type 5S rDNA repeats, only transcription of the oocyte-type gene surrounded by its native AT-rich flanks was repressed in the presence of physiological concentrations of histone H1 (8). We have now modified our DNA templates, so that the 120 bp oocyte 5S rRNA gene was placed in different positions relative to its native AT-rich flanking sequences (Fig. 2). The templates were then used for in vitro transcription in the form of both naked DNA and minichromosomes and with or without histone H1. To assess the effect of H1 on transcription, the RNA products were analysed on polyacrylamide gels. Consistent with our earlier results and with the results published later by others (17), transcription of the oocyte-type 5S rRNA gene surrounded by its AT-rich flanks was repressed by H1. In contrast, transcription
of the somatic-type 5S rRNA gene within its native GC-rich flanks was insensitive to H1 (Fig. 1B). However, H1 histone was unable to repress transcription of the oocyte 5S rRNA gene moved away from its original position within the AT-rich flanks by 546 and by 1237 bp (Fig. 2). We showed earlier that H1-mediated repression of transcription of the oocyte-type 5S rRNA gene is due to H1 selective binding to the AT-rich flanks. This leads to chromatin reorganization resulting in a decrease in the total number of nucleosomes in the minichromosome from 26 to 16 (Fig. 3A). In chromatin templates carrying the oocyte 5S rRNA gene separated by 546 (template II) and by 1237 bp (template III) from the AT-rich sequences, the total number of nucleosomes did not change after addition of H1 and was still 26 (Fig. 3B and C, respectively). Thus, no chromatin reorganization occurred.

We also checked the effect of H1 on transcription of the oocyte 5S rRNA gene located 546 bp away from an intact oocyte-type 5S rDNA repeat. To this end we inserted an additional copy of the 5S rRNA gene within native AT-rich flanks (template IV in Fig. 2B). In vitro transcription of this template in the form of both naked DNA and minichromosome without histone H1 yielded 5S rRNA product. However, the 5S rRNA product was absent in the presence of H1 (Fig. 2B). This argues that both 5S rRNA genes present in the template were subjected to H1-mediated repression. Thus, H1 is able to switch off the transcription of a 5S rRNA gene placed at a distance of 546 bp from the intact oocyte-type 5S rDNA repeat.

H1-mediated transcriptional repression of the distant 5S rRNA gene in the presence of the intact oocyte-type 5S rDNA repeat but not in the presence of AT-rich flanks alone could simply be due to a requirement for the separation of the flanks by a certain length of DNA. To test whether this is the case, we replaced (in template IV) the 120 bp 5S rRNA gene placed within native AT-rich flanks with a neutral 117 bp DNA fragment of pBR327 (template V in Fig. 2B). In vitro transcription of minichromosomes reconstituted on this template showed that histone H1 was unable to repress transcription of the 5S rRNA gene (Fig. 2B). Thus, the intact oocyte-type 5S rDNA repeat is absolutely required for H1-mediated transcriptional repression of the 5S rRNA genes in vitro.

The oocyte 5S rDNA repeat supports H1-mediated transcriptional repression of a pol II-type gene

We also performed an in vitro transcription assay with assembled nucleosome templates containing the region from –861 to +426 of the Drosophila Kr gene, transcribed in vivo by RNA polymerase II (Fig. 4). Transcription of the Kr gene from DNA template carrying this gene placed within the AT-rich flanks of the X.laevis oocyte 5S rRNA gene appeared to be slightly decreased after the addition of core histones and core histones with histone H1 (Fig. 4, left, lanes 4 and 5), as compared with transcription from the same template in the form of naked DNA (Fig. 4, left, lane 3). However, the decrease in the presence of histone H1 did not add up to the decrease already caused by core histones. In contrast, transcription of the Kr gene placed 546 bp from the intact oocyte 5S rRNA gene containing the AT-rich flanking sequences was completely repressed in the presence of H1 (Fig. 4, right, lane 5).

DISCUSSION

In our previous work (8) we found that a specific fragment of X.laevis DNA comprising the oocyte-type (but not the somatic-type) 5S rDNA repeat is capable of directing H1-mediated chromatin reorganization. This reorganization results in the removal of several already formed nucleosome cores from circular DNA, as well as in increased protection of DNA within the AT-rich oocyte 5S rDNA repeat. As the above structural effects were correlated with transcriptional repression of the oocyte 5S rRNA gene by
H1-mediated transcriptional silencing... AT-rich DNA sequences have to meet in order to direct the presented here was to define more precisely the conditions which oligo(dA)·oligo(dT) runs in DNA. The major aim of the work by distamycin A, a drug that specifically recognizes and binds oocyte 5S rRNA gene was indicated by their complete abolition selective binding to the AT-rich DNA sequences flanking the phenomenon (8). That all these effects of H1 were due to its H1, we suggested that they may be a direct cause of this minichromosomes consisting of reconstituted nucleosome core repression of the 5S rRNA gene upon addition of H1 to AT-rich sequences were unable to induce transcriptional AT-rich flanks and the oocyte 5S rRNA gene separated by a shorter or longer distance (Fig. 3). The lack of transcriptionally repressive chromatin structure on the 5S rRNA gene placed at a distance from the AT-rich flanks was not due to the fact that repression by H1 is a strictly local event that cannot spread away from the 5S rDNA repeat. The data of Figure 2B demonstrate that in the presence of an intact oocyte 5S rDNA repeat, H1-mediated repression spreads over a distance of at least 546 bp.

It could have been that the AT-rich flanks of the oocyte 5S rRNA gene could only induce H1-mediated repression when separated by a certain length of DNA. This possibility was ruled out by the results of the experiment in which the 120 bp 5S gene was replaced by a neutral fragment of DNA of a similar length (Fig. 2B). Thus, the phenomenon of H1-mediated repression of transcription of 5S rRNA genes in chromatin templates absolutely requires an intact oocyte 5S rDNA repeat.

The above results are consistent with the recent data of Sera and Wolffe (18). These authors have shown that the repression by histone H1 of transcription of an oocyte 5S rRNA gene assembled on a histone octamer results from H1-dependent restriction of the nucleosome position on the 5S rRNA gene to one that occludes the site of TFIIA access. The H1 binding sequence determining this effect of H1 was shown to be the AT-rich fragment extending from +123 to +146 to the 3’ of the gene. This fragment is a part of the AT-rich 3'-flank of the oocyte 5S rRNA gene. Thus, the sequence configuration that allows selective repression of oocyte 5S gene transcription by H1 requires that the 120 bp AT-rich 5S rRNA gene, known to position the nucleosome (19), and its AT-rich 3'-flank remain in close proximity. Sera and Wolffe suggest that except for the 21 bp AT-rich sequence identified by them in the 3'-flank, no other sequences in the flanks of the oocyte-type 5S rDNA repeat, especially those 5'-gene, are essential for the in vivo effects of H1. However, the results of Sera and Wolffe were obtained in experiments using short DNA fragments of a maximum length of 270 bp. The AT-rich sequence (CAAGGTGTTCAACTTTAATTGGC) they identified as binding H1 and allowing it to restrict the position of the nucleosome is repeated, forming most of the 5'-flank of the oocyte repeat, which we originally suggested as playing a key role in determining the differential effect of H1 on transcription of Xenopus 5S rRNA genes (11). It is difficult to imagine that the presence in the 5'-flank of the gene of multiple specific H1 binding motifs, identical to that in the 3'-flank, with a function in nucleosome positioning, would not contribute to the H1 effects in vitro. It would be particularly interesting to determine whether the oocyte gene AT-rich 5'-flank could stabilize the position of nucleosomes over the TFIIA binding site of the somatic type 5S rRNA gene.

Recently Panetta et al. reported substantial differences between multiple in vitro nucleosome positions on Xenopus somatic and oocyte 5S rRNA genes (20). In the competition between TFIIA and H1, TFIIA preferentially binds to nucleosomes on the somatic gene, whereas H1 preferentially binds to nucleosomes on the oocyte gene. These data indicate that the basis for the differential effect of H1 on the two types of Xenopus 5S rRNA genes could be even more complex and require input from both the AT-rich flanks and gene-specific nucleosome positioning.

**Figure 2.** In vitro transcription of the X. laevis oocyte-type 5S rRNA gene displaced from its original site. (A and B) Electrophoretic analysis of in vitro transcription products. DNA templates were in the form of naked DNA or assembled minichromosomes with or without H1, as indicated. Transcription was performed as described in Materials and Methods. Position of oocyte 5S rRNA is indicated.
Figure 3. Negative linking number change after minichromosome assembly without H1 and after addition of H1. After assembly the chromatin was incubated with excess topoisomerase I, deproteinized with proteinase K, extracted with phenol/chloroform and precipitated with ethanol. DNA was subjected to two-dimensional gel electrophoresis in 1.2% agarose as described (8). The DNA samples were co-electrophoresed with the standard DNA topoisomer mixture prepared according to Keller (21) and were loaded every 90 min into the same well in the order: chromatin with H1, chromatin without H1, standard. Arrows marked 1 and 2 in the left upper corner indicate directions of electrophoresis. Positions of nicked circular and linear plasmids are indicated by \( n \) and \( l \), respectively. Linking number change related to minichromosome assembly and thus reflecting the number of nucleosomes is calculated for each template. Analysis of minichromosomes reconstituted on pBR322 plasmids bearing the intact oocyte 5S rDNA repeat (template I) (A) and the oocyte 5S rRNA gene moved to a distance of 546 bp (template II) (B) and 1237 bp (template III) (C) from the AT-rich flanks.

Figure 4. *In vitro* transcription of the *Drosophila* *Kr* gene. Hybridization analysis of *in vitro* transcription products of the *Kr* gene in cell-free extract from mouse Ehrlich ascites cells. DNA templates were in the form of naked DNA (lanes 3) or assembled minichromosomes without (lanes 4) or with H1 (lanes 5), as indicated. Transcription was performed as described in Materials and Methods. The products of transcription were treated with RNase-free DNase, electrophoresed on a 1% agarose gel, transferred onto a nylon membrane (Hybond-N; Amersham) and hybridized at 68°C with a digoxygenin-labelled fragment of the *Kr* gene. Lanes 1 (both left and right), positive control; lanes 2, negative control; lanes 2a, *in vitro* transcription of *Drosophila Kruppel* gene (Promega Corp.); lanes 6, *in vitro* transcription with no DNA.

Binding of H1 to a single nucleosome over the oocyte 5S rRNA gene in the minichromosomes reconstituted *in vitro* on circular plasmids must have a crucial role not only in redistribution of the core particles in the adjacent DNA regions but also in the observed decrease in nucleosome density over the entire length of the plasmid DNA (the average length of DNA per nucleosome increases from 150 bp before addition of H1 to 240 bp in the presence of H1) (Fig. 3). In the absence of H1, the nucleosome positioning signal present in the 5S rRNA gene is clearly not sufficient for such a reorganization. One explanation of this puzzling long-range effect that seems to be dependent on the stabilization by H1 of a single nucleosome over the oocyte 5S gene could be that it initiates some form of a regular higher order structure requiring an optimum spacing of 240 bp for continuous (and presumably cooperative) spreading. A similar mechanism has been suggested to occur *in vivo* for the H1-dependent reorganization of chromatin over the oocyte 5S gene array (20).

The repression by H1 of transcription of the isolated oocyte 5S rRNA gene placed at 546 bp distant from the intact oocyte 5S rDNA repeat suggests that the position of the nucleosome on the distant 5S rRNA gene is identical to that on the 5S rRNA gene within the repeat, i.e. it occludes the TFIIIA binding site. However, the stabilization of the adjacent nucleosomes induced by binding of H1 to the oocyte 5S rDNA repeat is also sufficient to prevent transcription by polymerase II of the *Kruppel* gene, which lacks strong nucleosome positioning signals (Fig. 4). While we are aware that more experiments are needed to explain in detail the mechanism of the observed repression of pol II-type genes, the latter observation could be of practical importance for the manipulation of the activity of genes through targeted insertions of DNA fragments, like the *X.laevis* oocyte 5S rDNA repeat, capable of inducing local changes in the neighbouring chromatin status.

ACKNOWLEDGEMENTS

This work was supported by Howard Hughes Medical Institute grant 79195-543403 (A.J.) and Polish Committee of Scientific Research grants 6P04A 02913 (A.J.) and 6P04A02208 (R.T.).
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